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REGULAR ARTICLE

Soil temperature limits nitrogen fixation, photosynthesis, and growth in a boreal actinorhizal shrub

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Abstract
Purpose The short growing season and cold climate of the boreal forest can restrict soil nitrogen availability, limiting plant growth and ecosystem productivity. Vascular nitrogen-fixing plants should have an advantage in low nitrogen environments. Yet, their abundance in the boreal forest is low. How nitrogen fixation is affected when temperature differences occur between the soil and air, especially in the spring when soil temperatures remain cool, has not been documented in actinorhizal shrubs.
Methods A lab study was performed on *Alnus alnobetula* subsp. *crispa* (Aiton) Raus. For 13 weeks, soil was kept at either 10˚C, 14˚C or 16˚C, independently of shoot temperature, at 20˚C.
Results Soils at 14˚C and 10˚C inhibited whole-plant nitrogen fixation (by 53% and 68%) and photosynthesis (by 43% and 39%), respectively, compared to soils at 16˚C. Reductions in photosynthetic rate were mainly attributed to a reduction in the fixed nitrogen supply and subsequent reduction in chlorophyll formation. Photosynthesis was not reduced immediately, suggesting some utilization of a nitrogen source not supplied from current fixation. Reduced amounts of fixed nitrogen and photosynthates resulted in diminished biomass production and relative growth rate.
Conclusion The assumed advantages of being a nitrogen-fixing plant in a low nitrogen environment may be constrained by soil temperature to a larger extent than previously considered. This may restrict the abundance of nitrogen-fixing species in the boreal forest.

Keywords *Alnus crispa* (green alder) · Boreal forest soil · *Frankia* · Nitrogen fixation

Introduction

The degree to which nitrogen limits an ecosystem's net primary productivity increases with latitude (McGuire et al. 1992), with little nitrogen limitation in many areas of the tropics (Brookshire et al. 2012). Regions that experience cool temperatures, low precipitation or both, have lower soil mineralization rates when compared to regions with warmer and wetter climates (Devito et al. 1999; Jerabkova et al. 2006; Neill et al. 1997; Raghubanshi 1992). This results in the limited availability of inorganic soil nitrogen. Additionally, the atmospheric deposition of anthropogenic nitrogen in the boreal forest can vary depending

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on population density and is generally lower in the North American boreal forest (approximately 2 kg N/ha/year) than in the European boreal (approximately 60 kg N/ha/year, Dentener et al. 2006). While some plants can utilize simple forms of organic nitrogen (amino acids) present in the soil, and the uptake of organic N is often described as short-circuiting the mineralization process (Näsholm et al. 2009), there is no evidence that plants take up complex forms of organic nitrogen. Therefore, the mineralization of organic material is still likely to limit plant productivity in cold and dry climates.

The uptake of both inorganic and organic nitrogen can be influenced by soil temperature. For example, in ryegrass (*Lolium* spp.), nitrate and ammonium uptake was reduced at temperatures lower than 14˚C and 10˚C, respectively (Clarkson and Warner 1979). In birch (*Betula pubescens*), reductions in overall root nitrogen uptake in field trials were observed with decreasing soil temperature (Weih and Karlsson 1999). In *Cerastium alpinum* and *Saxifraga caepitosa,* species which inhabit a polar desert, long-term organic and inorganic nitrogen absorption was reduced at 6˚C, compared to 15˚C soil temperatures (Volder et al. 2000). Thus, in the boreal forest both the production and uptake of nitrogen can limit plant growth and ecosystem productivity.

In terrestrial ecosystems, nitrogen input can occur through nitrogen fixation by heterotrophic and autotrophic prokaryotes (Markham 2009; Stal 2015). In addition, a restricted clade of vascular plants also can form nitrogen-fixing nodules in their roots via symbiosis with specialized bacteria (Mylona et al. 1995), with rates of nitrogen fixation being high on a per mass basis compared to other terrestrial, non-symbiotic, nitrogen-fixing systems (Boring et al. 1988). Symbiotic nitrogen fixation allows nitrogen-fixing species to develop independently of soil nitrogen availability (Markham and Zekveld 2007). Since it is more energetically costly than the uptake of nitrogen (Lundquist 2005; Silsbury 1977), the fixation of atmospheric nitrogen should be most beneficial in areas of limited inorganic soil nitrogen availability. However, the global distribution of nitrogen-fixing plants does not match this prediction. The abundance of nitrogen-fixing plants decreases as soil inorganic nitrogen availability becomes more limited, i.e., from low to high latitudes. Additionally, the composition of nitrogen-fixing species shifts from mainly leguminous trees in tropical regions to mainly actinorhizal shrubs (which form a symbiotic relationship with actinobacteria *Frankia* spp.) in boreal forests (Menge et al. 2014). In higher latitudes, nitrogen-fixing vascular plants are also generally only abundant in early successional habitats (Menge et al. 2010). Although actinorhizal shrub can be found throughout the North American boreal forest, and even above the tree line, there are no nitrogen-fixing tree species in this region. The higher abundance of nitrogen-fixing plants at lower latitudes has been explained by environmental constraints that would, in theory, increase the cost of fixation beyond limits that are beneficial to the host plant, resulting in a reduction in nitrogen fixer abundance (Vitousek and Howarth 1991). Environmental factors have been used to explain the lack of success of nitrogen-fixing plants as they can limit the development of the nodules and activity of the nitrogenase enzyme. These include increased availability of inorganic soil nitrogen (Markham and Zekveld 2007), a higher demand for light (Rastetter et al. 2001; Vitousek et al. 2002), phosphorus availability (Gentili et al. 2006) and temperature (Houlton et al. 2008).

The effect of colder soil, compared to air temperatures, on symbiotic nitrogen fixation and the consequential distribution of nitrogen-fixing plants in natural ecosystems has received little attention. Increased soil temperature is known to increase plant growth and nitrogen content in non-fixing plants, similar to the effects of increased nutrient availability (Weih and Karlsson 2001). The optimum temperature for the nitrogenase enzyme (in symbiosis) is generally considered to be around 25˚C but can be as high as 42˚C (Houlton et al. 2008; Waughman 1977). Soils in the boreal forest are typically well below this level, especially in the first part of the growing season when soil temperatures can be substantially lower than air temperatures (Fig. 1). On a global scale, the mismatch between air and soil temperatures is particularly pronounced in the spring in higher latitudes (Lembrechts et al. 2021). This discrepancy in above- and below-ground temperatures leads to a mismatch between

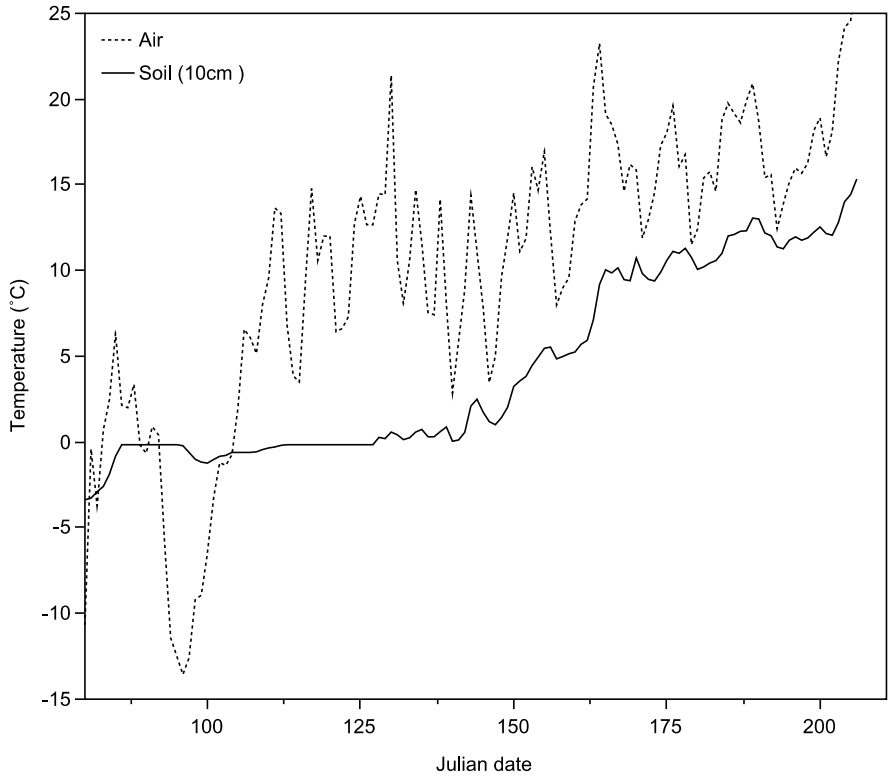

Fig. 1 Spring and summer mean daily air and soil temperatures from a southern boreal forest *Pinus banksiana* stand (51°10' N, 95°54' W) with *Alnus crispa* growing in the understory. Soil temperatures are from a depth of 10 cm, where nitrogen-fixing nodules are typically found. At this site *A. crispa* buds opened on day 155

photosynthesis and nitrogen fixation. Soil warming during the spring may be further delayed in areas with ground cover, such as moss, which can act as an insulator (Startsev et al. 2007). This may decrease the rate at which frozen soils thaw and begin to warm during the spring, additionally delaying the start of nitrogen fixation. To our knowledge, no controlled laboratory experiments have been conducted on the effect of the differences in root and shoot temperatures on nitrogen fixation in actinorhizal shrubs. However, Larigauderie et al. (1991) did measure the effect of root and shoot temperature on the growth of a non-nodulated actinorhizal plant.

Unlike herbaceous species, the nitrogen-fixing nodules of actinorhizal plants are perennial and so do not have to reform each year. Previous field studies in boreal and northern temperate forests have shown that nitrogen fixation in actinorhizal shrubs becomes detectable in the spring, peaks in mid-summer, and ceases around the time of leaf senescence in the fall (Markham and Anderson 2021; Huss-Danell et al. 1992). In the fall, the re-absorption of nitrogen from senescing leaves is essential for many deciduous species to conserve nitrogen. However, in many actinorhizal species nitrogen re-absorption is low compared to non-fixing species (Kimmins 1987). Therefore, nitrogen-fixing plants could have less stored nitrogen for growth in the spring compared to non-nitrogen fixing plants. Consequently, cold soils in the spring could be particularly problematic for nitrogen-fixing plants in boreal forest habitats.

The objective of this study was to investigate how soil temperature, independent from air temperature, affects nitrogen fixation and how this, in turn, affects whole plant physiology and growth in the actinorhizal shrub *Alnus alnobetula* subsp. *crispa* (Aiton) Raus. (hereafter referred to as *A. crispa*). We predict low soil temperature will cause a reduction in the activity of nitrogen fixation, making plants more nitrogen limited. This will lead to reductions in above-ground photosynthesis and lower overall growth compared to *A. crispa* growing in warmer soils. We tested this idea using an environmental chamber where we controlled soil temperature independently of air temperature in young *A. crispa* seedlings.

Materials and methods

Experimental set-up

Alnus crispa seeds were collected from at least a dozen individual shrubs in a population located in Beaver Creek, Manitoba, Canada (51°09′49.4 "N 95°53′47.5 "W). Seeds were stratified at 6˚C for one month and then sown on a commercial peat moss and perlite mixture (Sun Gro Horticulture, Canada). Plants were then transferred to 2.9 L pots with mineral soil obtained from a variety of locations in the Sandilands Provincial Forest, Manitoba, Canada (49°22′58.2 "N 96°12′56.6 "W) at a depth of approximately 20–30 cm, in which *A. crispa* was already present. Soils were low in inorganic nitrogen (ca. 7 mg kg^{-1}), measured on 2 M NaCl extracts using the microdiffusion method (Khan et al. 2000), and soil phosphate (ca. 1 mg kg^{-1}) measured on Bray extracts. The soil consisted mainly of sand, with a pH of 6.6. Plants were initially grown under artificial fluorescent light with a 16-h photoperiod at a constant temperature of 21˚C. Seedlings were inoculated with crushed *Frankia* sp. nodules obtained from *Alnus rugosa* (acquired from the Sandilands Provincial Forest, Manitoba, Canada) after two months of growth. Seedlings were fertilized with Rorrison's nutrient solutions (Booth et al. 1993). A modified (1 mM nitrate) nutrient solution was applied two times before *Frankia* inoculation to allow for seedling growth. An N-free Rorrison's nutrient solution was applied once after inoculation. Plants were watered as needed with distilled water. After five months, plants were forced into dormancy by placing them in an environmental room set at 10˚C with a 10-h photoperiod for three weeks and were then moved to a cold room at 5˚C, with no light. All plants lost their leaves, and buds were fully formed. The dormancy period lasted for six weeks. At the start of the experiment, the plant height and diameter were 6.16 ± 1.60 cm and 2.3 ± 0.33 mm (mean ± standard deviation), respectively.

For the experimental treatments, plants were placed in an environmental room set at 10˚C with 400 W full spectrum plasma lights. Four plants were paced into each of 15 boxes. The boxes were 43 cm by 43 cm and built to thermally isolate the roots from the shoots (Fig. 2). The bottom section of the boxes was made of plywood and was 15 cm tall. The top of the boxes was made from polycarbonate (Thermoclear^{tm} Lexan^{tm}, Sabic Plastics Inc) and was 30 cm tall. Light levels in the upper section of the boxes varied from 80 to 100 µmol m^{-2} s^{-1} PAR. These light levels are typical of understory light levels where we find *A. crispa*. The bottom and top sections isolated the roots from the shoots using an insulating foil-lined bubble wrap (Reflectix Inc., USA). Microcontrollers (Arduino, Italy) were used to manipulate the soil and shoot surrounding temperatures independently.

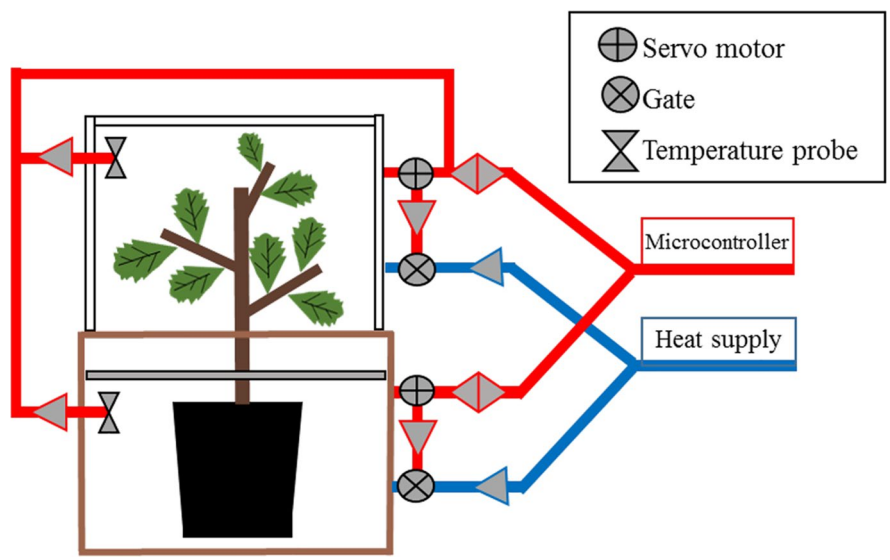

Fig. 2 Box system to separate shoot and roots. The top section is made of Lexan plastic (shown in white), and the bottom section of plywood (shown in brown). Sections were separated with insulating bubble wrap (shown in grey). Red lines indicate wires. Blue lines represent warm air ducts. Image shown with one pot. The actual system contained four pots per box

Temperature sensors connected to the microcontrollers were placed in the upper and lower sections of the boxes. Motor actuated vents allowed for warm air (supplied from an electrical heating cable and fan) to keep the boxes at their designated temperatures. The air supply to the heating system was from the growth chamber. Plants were watered via tubing connected to each pot. Plants were watered two to three times per week using distilled water. Additionally, plants were watered prior to nitrogen fixation and photosynthesis measurements. Plants were fertilized with nitrogen-free Rorrison's nutrient solution at the beginning, and halfway through (week six), the experimental period. The boxes were divided into three root temperature treatments: 10˚C, 14˚C, and 16˚C, with five replicate boxes per treatment. These values were selected based on preliminary temperature data collected from the site where the seeds were collected (Fig. 1). Shoot temperature was maintained between 20˚C and 22˚C throughout the experimental period in all treatments. The experimental treatments lasted 13 weeks, typical of a growing season in this area of the boreal forest.

Biomass and growth

Plant height and diameter (taken from the base of the plant) were measured every other week. At the end of the experiment, plant tissues were freeze-dried and weighed. A least squares fit was used to calculate the relationship between total dry mass and height of the plants, multiplied by the diameter squared at the time of harvest (Supplemental Fig. 1). This relationship was then used to estimate plant mass over the course of the experiment. Relative growth rate was then calculated as the difference in the log mass between the start of leaf development (week four) and the end of the experiment (Hunt 1978).

Acetylene reduction assay

Nitrogenase activity was measured on every plant while the plants were in dormancy (week zero), and every second week up to and including harvest (week 13), using acetylene reduction assays (Hardy et al. 1968). Measurements were made between 10 am and 4 pm. Pots of whole plants were sealed in glass boxes lined with insulating bubble wrap to maintain the soil temperature treatments during analysis. Acetylene gas was injected at 12–16% headspace volume. After one hour, a 5 mL sample of gas was collected and analyzed in a Varian 3400 gas chromatograph (Varian, Canada) fitted with a 0.25 mL sample valve and a Haysep T column (Markham and Zekveld 2007). Nitrogenase activity was expressed on a whole plant mass basis using the estimate of plant mass at each sampling time, as stated above. To express nitrogenase activity on a nodule mass basis, nodule mass was estimated at each sampling time. This required estimating nodule mass over time, and we assumed a constant biomass allocation to nodules throughout the experiment (Supplemental Fig. 2) relative to whole-plant mass.

Photosynthetic rate

The photosynthetic rate was measured on every plant every two weeks (starting at week six, when all plants had fully expanded leaves) using a Li-6400 infrared gas analyzer (Licor, USA). Measurements were conducted for 5 min at a CO_2 flow rate of 400 µmol/s, a temperature of 25 °C, relative humidity of 33%, and a light level of 700 µmol m^{-2} s^{-1} PAR, to ensure photosystem light saturation. Measurements were made on the second mature leaf from the top (or the first mature leaf if a second leaf was not developed) between 10 am and 3 pm. Stomatal conductance was synchronously measured with photosynthetic rate, using the same equipment, to determine if the temperature treatments would affect water use by the plants.

Leaf analysis

Chlorophyll content of the leaves was measured at the end of the experimental period by taking 20 mg of powdered dry leaf sample (homogenized from all leaves) in 8 mL of methanol and left overnight. The following day samples were centrifuged at 14,500 g (Sorvall Legend 14, ThermoFisher Scientific, USA) and their absorbance was read with an Ultrospec 2100 pro spectrophotometer (Biochrom, USA) at 650 nm and 665 nm. The MacKinney equations were used to determine chlorophyll concentration (Sestak et al. 1971).

Powdered dry leaf samples were sent to UC Davis Stable Isotope Facility and measured for stable nitrogen ($\delta^{15}N$) and carbon isotope ($\delta^{13}C$) analysis. The amount of nitrogen derived from fixation (%Ndfa)

was determined using the following formula from Andrews et al. (2011):

$$\frac{\delta^{15}N(\text{Reference soil from field}) - \delta^{15}N(\text{Nitrogen fixing plant})}{\delta^{15}N(\text{Reference soil from field}) - B} * 100 = \text{Ndfa}(\%)$$

where B is the δ^{15} N of A. crispa grown on N free (acid washed) Turface (Supplementary Table 1). Carbon isotope abundance values were used to calculate carbon discrimination according to Farquhar et al. (1989) to examine the effect of soil temperature on stomatal closure. The atmospheric carbon $\delta^{13}C$ value for air was set at -0.008‰.

Statistical analysis

Statistical tests were performed on the mean values of the four plants in each temperature control box. The effect of soil temperature on plant performance was examined using least squares models with the temperature set as a continuous effect. For response variables measured over time, repeated measures models were also run, using the box as a random variable. We also ran least squares mixed models for each sampling time separately. Nitrogenase activity values were analyzed with a log (+ 1) transformation in all analyses. All analyses were performed using JMP Pro 14 (SAS Institute, USA).

Results

Increasing root temperature resulted in both increased physiological activity and overall plant growth. At the start of the experiment, there was no detectable nitrogenase activity in any of the plants. Plants started showing nitrogenase activity two weeks after being brought out of dormancy (Fig. 3), before leaves had fully expanded. A repeated measures analysis for weeks two to 13 showed that nitrogenase activity per plant mass significantly increased with increasing root temperature (Supplementary Table 2) and significantly increased over time, but there was no interaction between temperature and sampling time. On average, between week two and harvest (week 13), the 10˚C treatment had 68% less nitrogenase activity than the 16˚C treatment, and there was 53% less nitrogenase activity in the 14˚C treatment compared to the 16˚C treatment. When compared within each sampling period, nitrogenase activity per plant mass significantly increased with temperature at week four (

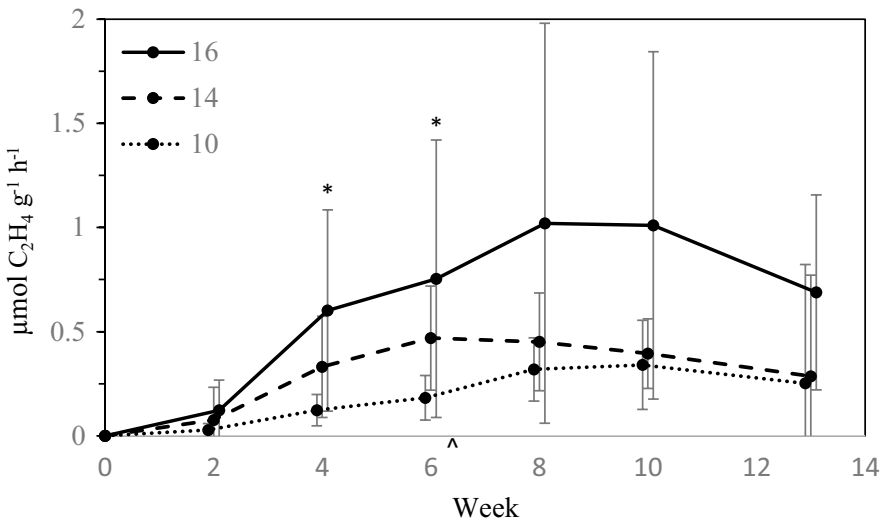

Fig. 3 Nitrogenase activity per plant mass of *Alnus crispa* nodules measured using acetylene reduction assay. Measurements began while plants were in dormancy (week zero). Plants were exposed to different root temperatures (10 °C, 14 °C, 16 °C) while the shoot temperature remained at 20 °C. Points are means (n = 5, with 4 subsample plants per replicate) with standard deviation error bars.. * Indicates weeks when there were significant differences between treatments. ^ Indicates time of leaf maturity in all treatments

$F_{1,13}=6.5173$, $p=0241$) and week six ($F_{1,13}=6.0884$, $p=0.0283$) with increases of 0.0759 and 0.0914 µmol C_2H_4 g^{-1} whole-plant mass h^{-1} for every degree increase in soil temperature, respectively. No significant differences in nitrogenase activity were observed at week two ($F_{1,13}=1.5653$, $p=0.2329$), week eight ($F_{1,13}=3.1055$, $p=0.1015$), week ten ($F_{1,13}=3.6911$, $p=0.0769$), or at harvest ($F_{1,13}=3.8753$, $p=0.0707$). When nitrogenase activity was expressed on a nodule mass basis, similar trends were found. There was a significant increase in nitrogenase activity with root temperature and with time, but no interaction between temperature and time. When these data were analyzed separately at each time period there were significant positive effects of root temperature at week four ($F_{1,13}=10.7054$, $p=0.0061$), week six ($F_{1,13}=12.9910$, $p=0.0032$) and week eight ($F_{1,13}=14.9346$, $p=0.0020$), but not at week two (1.1877, $p=0.2956$), week 10 ($F_{1,13}=3.4179$, $p=0.0874$) or at harvest ($F_{1,13}=2.8557$, $p=0.1149$). The increasing nitrogenase activity with root temperature resulted in the plants deriving significantly more nitrogen from fixation ($F_{1,13}=10.9610$, $p=0.0057$), measured on leaves at the time of harvest (Table 1).

By week six, all plants had developed mature leaves. From week six to the end of the experiment, there was no overall effect of root temperature on the photosynthetic rate, which significantly declined over time. However, when the data was analyzed for individual weeks, root temperature significantly

Table 1 Biomass, relative growth rate (RGR), leaf chlorophyll, nitrogen derived from fixation (Ndfa), and carbon discrimination of *Alnus crispa* plants (mean ± SE, n = 5, with 4 subsample plants per replicate). Measured at harvest (week 13) under different root temperature treatments (10 °C, 14 °C, and 16 °C) and a consistent shoot temperature (20 °C)

	Soil Temperature (˚C)			$F_{1,13}$	p Value
	16	14	10		
Chlorophyll a (mg g^{-1})	4.63 ± 0.25	4.01 ± 0.39	3.34 ± 0.12	11.1207	**0.0054**
Chlorophyll b (mg g^{-1})	1.68 ± 0.11	1.52 ± 0.19	1.29 ± 0.18	3.1033	0.1016
Plant dry biomass (g)	1.65 ± 0.22	1.16 ± 0.16	0.98 ± 0.10	7.5974	**0.0164**
RGR (mg g^{-1} $week^{-1}$)*	22.0 ± 3.26	13.9 ± 2.88	8.86 ± 2.90	8.6556	**0.0114**
Root:Shoot	0.79 ± 0.06	0.94 ± 0.03	0.95 ± 0.03	3.4579	0.0857
Ndfa (%)	88.8 ± 2.17	83.4 ± 2.55	77.7 ± 2.48	10.9106	**0.0057**
Leaf C:N	27.7 ± 0.56	30.4 ± 1.22	34.1 ± 0.69	28.1743	**0.0001**
$Carbon^{13}$ discrimination (‰)	25.1 ± 0.26	24.7 ± 0.36	24.1 ± 0.25	7.0328	**0.0199**

Significant p values are in bold

* Between week four and week thirteen

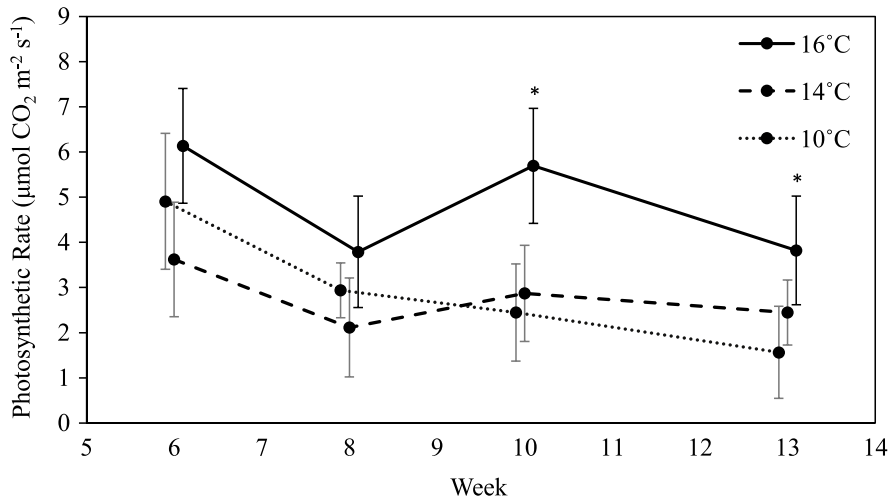

Fig. 4 Photosynthetic rate of *Alnus crispa* over time. Plants were exposed to different root temperatures (10 °C, 14 °C, 16 °C) while the shoot temperature remained at 20 °C. Samples were measured on the second mature leaf at 700 µmol photons m^{-2} s^{-1}. Mean ± SE. n = 5, with 4 subsample plants per replicate. n = 3–5 for week six

increased photosynthesis in weeks ten ($F_{1,13}=6.37$, $p=0.0259$) and 13 ($F_{1,13}=6.81$, $p=0.0221$, Fig. 4), with increases of 0.48 and 0.36 µmol CO_2 m^{-2} s^{-1} for every degree increase in soil temperature at weeks ten and thirteen, respectively There were no significant differences in photosynthetic rate between the treatments at week six ($F_{1,13}=0.3701$, $p=0.5535$) and eight ($F_{1,13}=0.5551$, $p=0.4695$). On average, between week six and harvest (week 13), 39% less photosynthetic activity occurred in the 10˚C treatment and 43% less activity in the 14˚C treatment, when compared to the 16˚C treatment. When analyzed over the whole experimental period, root temperature had a significant positive effect on stomatal conductance. When each sampling period was analyzed separately, leaf stomatal conductance significantly increased with root temperature at week ten ($F_{1,13}=10.5229$, $p=0.0064$), increasing by 0.167 mol H_2O m^{-2} s^{-1} for every degree increase in temperature (Table 2), but did not differ with temperature at any other sampling period.

At harvest, dry tissue mass increased with increasing root temperature (Table 1). Compared to the 16˚C treatment, total dry mass was reduced by 30% in the 14˚C treatment and 41% in the 10˚C treatment. No change in nodule allocation or root to shoot ratio was observed. The relative growth rate was 37% lower in the 14˚C and 60% lower in the 10˚C soil temperature treatment, compared to the 16˚C treatment. The chlorophyll content was significantly increased for chlorophyll a, but not chlorophyll b, with increasing root temperature (Table 1). The ratio of chlorophyll a:b was not significantly different between treatments (data not shown). The carbon to nitrogen ratio of the leaves was significantly higher at lower root temperatures, increasing by 9% at 14˚C and 19% at 10˚C root temperature compared to the 16˚C treatment. Carbon isotope discrimination was reduced with low root temperature by 2% and 4%, at root temperatures of 14˚C and 10˚C, respectively.

Table 2 Stomatal conductance (mol H_2O m^{-2} s^{-1}; mean ± SE, n = 5, with 4 subsample plants per replicate) at individual weeks, of *Alnus crispa* plants under different root temperature treatments (10 °C, 14 °C and 16 °C) and a consistent shoot temperature (20 °C)

	Soil Temperature (˚C)			*p* Value
	16	14	10	
Week 6	1.34 ± 0.14	0.68 ± 0.14	0.98 ± 0.27	0.4285
Week 8	0.89 ± 0.22	0.42 ± 0.06	0.65 ± 0.16	0.5398
Week 10	1.66 ± 0.30	0.78 ± 0.14	0.55 ± 0.11	**0.0064**
Week 13	1.10 ± 0.08	0.98 ± 0.14	0.67 ± 0.21	0.0510

Significant *p* values are in bold

Discussion

These results demonstrate that lower soil temperatures, compared to air temperature, typical of boreal climates, limits nitrogen fixation activity, photosynthesis, and growth in *Alnus crispa,* one of the most common nitrogen-fixing species in the North American boreal forest. Prior to budburst, nitrogen fixation remained minimal for all plants. During this period, a lack of carbohydrate production to support the high cost of nodule function (Lundquist 2005) likely restricted nitrogen fixation in all plants, regardless of soil temperature. Once new leaves expanded, nitrogen fixation was reduced in colder soils for most of the growth period. In general, enzyme kinetics and metabolic activity slows at low temperatures (Gillooly et al. 2001). As such, all aspects of root and soil microbial activity should decrease in cold soils, reducing root activity and soil nutrient availability. In non-fixing plants, this can lead to decreased shoot growth and decreased leaf nitrogen content due to reduced soil nitrogen uptake (Karlsson and Nordell 1996). In addition, lower soil temperature has been shown to increase the activation energy and energetic cost associated with the nitrogenase enzyme (Duke et al. 1979; Winship and Tjepkema 1985). Therefore, cold soils increase the already high cost associated with nitrogen fixation and could therefore make symbiosis less advantageous to the host plant as a strategy for nitrogen acquisition. This would help to explain the lack of success of nitrogen-fixing plants outside of early successional habitats in the boreal forest.

Although reduced soil temperatures reduced overall plant growth, biomass allocation to nodules was not affected. This was likely because actinorhizal nodules are perennial structures (Huss-Danell 1990) and the nodules in this study formed the previous growing season. In herbaceous legumes, where nodules are formed annually, nodule formation and growth are reduced at temperatures below 17˚C (Zhang and Smith 1994). Actinorhizal plants are known to have

the ability to modify nodule allocation in response to other environmental variables, such as increased soil inorganic nitrogen availability (Markham and Zekveld 2007). Therefore, the effect of repeated annual delays in soil warming on nodule allocation requires investigation.

Lower soil temperature was anticipated to reduce the amount of fixed nitrogen, resulting in a decrease in the formation of nitrogenous compounds such as chlorophyll and consequently reducing photosynthetic activity. However, reduced photosynthesis under lower soil temperatures only occurred toward the end of the growth period, four weeks after leaf emergence. The herbaceous annual plant soybean (*Glycine max*) showed a reduction in nitrogen fixation and photosynthesis by half, within two days of roots being cooled from 20˚C to 13˚C (Duke et al. 1979). The perennial nature of our plants may explain our lack of temperature effect on photosynthesis early in the experimental period. *Alnus crispa* coming out of dormancy would potentially have access to a nitrogen supply stored during the previous growth period. Non-fixing woody species often utilize stored nitrogen in the spring for new leaf development. For example, Millard (1994) found that in maple (*Acer pseudoplatanus*), a third of nitrogen in newly developed leaves in the spring was derived from overwintering stored nitrogen. Since actinorhizal species do not reabsorb large amounts of leaf nitrogen, compared to non-actinorhizal species (Dawson and Funk 1981) and there is little increase in stored nitrogen as actinorhizal plants enter dormancy (Kurdali 2000), it is likely that actinorhizal shrubs, including *A. crispa*, have a limited capacity to sustain early seasonal photosynthesis and growth from stored nitrogen. Because all our plants went into dormancy after growing in the same growth conditions, if they were relying on stored nitrogen, they most likely would have similar values of stored nitrogen, regardless of imposed treatment conditions.

By limiting nitrogen fixation activity and thus supplied nitrogen, reducing soil temperature inhibited photosynthesis and relative growth. The low soil temperatures also led to decreased stomatal conductance and carbon discrimination, indicating some level of water stress in the plants (Farquhar et al. 1989). Stomatal closure is a common response to reduced soil temperature (Landhäusser et al. 1996, Cox and Boersma 1967). Lowered soil temperatures can impede root elongation (Andersen et al. 1986), which may consequently reduce water uptake. Additionally, the viscosity of water increases, and membrane permeability decreases as temperatures decline (Kuiper 1964), further impeding water uptake. A study with well-fertilized, non-inoculated *A. crispa* found no effect of soil temperature on photosynthetic activity or stomatal conductance (Lawrence and Oechel 1983). This suggests that in our experiment, while stomatal closure inhibited photosynthesis at lowered root temperatures to a small extent, a lack of nitrogen derived photosynthetic pigments likely had a greater impact.

The reduced nitrogen fixation per plant mass resulted in plants obtaining less nitrogen from fixation than plants at warmer soil temperatures. This shift to increased soil uptake of inorganic nitrogen means that *A. crispa* would supply less fixed nitrogen to the ecosystems, which could limit ecosystem productivity. These effects are likely to be more accentuated with the more mature plants typically found in the forest understory since their nodules tend to be found deeper in the soil, which would take longer to warm in the spring. In boreal systems, the lack of nitrogen-fixing plants may be partly offset by nitrogen fixation associated with mosses, which can contribute a substantial proportion of the nitrogen budget to the system (Markham 2009). However, moss associated nitrogen fixation may be particularly sensitive to small inputs of nitrogen from atmospheric sources (Gundale et al. 2011).

The energetic cost of nitrogen fixation has been used to explain the lack of nitrogen-fixing plants in temperate and boreal systems (Houlton et al. 2008). Additionally, the climate has been used to explain the low abundance of nitrogen-fixing trees in high latitude forests (Steidinger et al. 2019). This is the first study to explicitly address the problem of cold soil in boreal forest systems. Our results suggest that the energetic costs associated with fixing nitrogen are most likely intensified when the soil remains cool in the spring, limiting the role of nitrogen fixation in the boreal forest. Our results show that reductions in soil temperatures decreases nitrogen fixation, photosynthesis, and chlorophyll production, leading to declines in growth and biomass production. This may contribute to the limited availability of nitrogen-fixing shrubs in the boreal forest compared to warmer ecosystems. Even though *A. crispa* have perennial

nodules, which should allow for fixation to occur in the spring without having to first put time and energy into the production of new nodules, soil temperatures in boreal regions may limit this possible benefit over herbaceous fixers and non-fixing species.

Long term studies of increased carbon dioxide in natural systems have shown that the increased carbon content of leaf litter results in decreased soil nitrogen availability. This can favor nitrogen-fixing plants (Field et al. 2007) and increase N fixation if other elements are not limiting (Hungate et al. 2004). Our results suggest that warmer soils in regions of the boreal forest, where nitrogen limits plant growth, will give nitrogen-fixing plants a further competitive advantage.

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Code availability Not applicable

Declarations

Conflicts of interest/Competing interests The authors declare they had no conflict of interest or competing interests.

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